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13. ABSTRACT (Maximum 200 Words)

GADD34 is a stress-induced proteins implicated in the control of protein synthesis and apoptosis. It is a major target of the oncogene c-myc. Myc is widely implicated in breast cancer and potently inhibits GADD34 expression. The goal of this proposal is to better understand the mechanism of GADD34 induced apoptosis and the implications of this to human breast cancer. Toward this goal we have analyzed the regulation of eIF2 alpha dephosphorylation by GADD34 and by its binding partner Inhibitor-1. We have demonstrated that proper subcellular localization of GADD34 is necessary for function. We have identified a novel domain that targets GADD34 to the endoplasmic reticulum, and a domain involved in PP1 binding. We also examined the role of I-1, a GADD34 interacting protein that inhibits PP1, in inhibiting eIF2 alpha dephosphorylation and identified a novel domain necessary for the *in vivo* function of I-1. We also demonstrated that this domain is absent in 2 alternate splice forms of I-1, I-1 alpha and I-1 beta, which are weaker inhibitors of eIF2 alpha dephosphorylation. We have also demonstrated that GADD34 protein levels are elevated in human cancer cells in response to a variety of stressed. Interestingly, p38 MAP kinase is required for GADD34 induction by aresnite, but not endoplasmic-reticulum stress. GADD34 is also a rapidly degraded protein, consistent with a temporal regulation of stress-signaling. This work has lead to a better understanding of GADD34 function in cancer cell, and may lead to better anti-breast-cancer drugs targeting this apoptotic pathway.

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Introduction:

The proto-oncogene c-myc is highly expressed in many human breast cancers. Among its many actions, c-myc suppresses the expression of GADD34 (Amundson et al, 1998), an apoptosis-associated gene, in response to DNA-damaging agents and UV irradiation. The anti-cancer compound methylselenocyteine (MSC) induces GADD34 and promotes cell death in cancer cells (Sinha et al, 1999). Thus, we hypothesize that understanding the expression and function of GADD34 in human breast cancer cells may provide new avenues for assessing the efficacy of anti-cancer therapy and promote the design of more effective therapies against human breast cancer. The primary cellular role identified for GADD34 is to induce the dephosphorylation eIF2 alpha. Phosphorylation of eIF2 alpha inhibits overall translation, but the translation of some mRNAs, especially stress-response transcription factors, is increased. This activates a stress-response cascade that allows the cell to recover from stress. If this pathway is misregulated, either by prolonged activation, or inhibition, apoptosis can occur.

Our recent studies demonstrated that GADD34 scaffolds the PP1 catalytic subunit and an endogenous PP1 inhibitor (Connor et al, 2001), known as inhibitor-1 (I-1). PP1 and I-1 associate with distinct non-overlapping sites in GADD34, allowing for effective PP1 regulation by I-1. Thus, we hypothesize that the regulated assembly of the PP1/GADD34/I-1 complex allows phosphorylated I-1 to inhibit the activity of GADD34-bound PP1 and promote eIF2 alpha phosphorylation and apoptosis. Understanding the mechanisms that dictate the expression of GADD34 and the assembly of the cellular complex containing PP1 and I-1 in human breast cancer cells may be crucial for the design of improved anti-breast cancer therapies.

Body:

Our statement of work was divided into two parts seeking to understanding the mechanism of GADD34 regulation of apoptosis. **Task 1** is to understand when GADD34 is induced in breast cancer cells and whether this induction induces apoptosis and if this apoptosis is dependent on PP1 and eIF2 alpha. **Task 2** is to understand the mechanism of GADD34 regulation in breast cancer cell lines. Significant progress has been made toward both **Task 1** and **Task 2** in the last year. This progress has lead to the submission of one paper (Weiser et al. 2004).

Specifically toward **Task 1**, we have conducted numerous preliminary experiments to study the role of induction of GADD34 by c-Myc and other signals as described in **Task 1A**. We have monitored GADD34 levels using RT-PCR and immunoblot analysis to assay mRNA and protein levels respectively. Most previous studies of GADD34 induction have focused on mRNA since most available GADD34 antibodies are quite poor (Hollander et al, 2001). We have obtained a GADD34 antibody that will recognize human GADD34 well in both immunoblots and immunocytochemistry. Thus we have undertaken the most detailed analysis of GADD34 protein induction in human cancer cells to date. Previous work has shown that GADD34 mRNA is rapidly up-regulated by a variety of stresses, but that the level of mRNA drops as the stress is relieved (Hollander et al, 2001). This short induction of GADD34 is required for proper regulation of stress-signaling (Novoa et al, 2003). If, however, GADD34 is up-regulated for a prolonged period of time, such as when cells are treated with MSC, the cells will undergo apoptosis (Hollander et al, 2001).

We have studied the induction of GADD34 in several cell lines in response to various stresses, including endoplasmic reticulum overload, okadaic acid treatment, DNA damage, osmotic stress, oxidative damage and arsenite toxicity. We then used this system to determine that multiple pathways act upstream of GADD34 induction. Inhibition of the p38 MAP kinase pathway blocked induction of GADD34 in response to arsenite, but not ER-stress in all cell types. We also detected cell-type specific regulation of GADD34 expression. Arsenite treatment was the most potent inducer of GADD34 protein in HEK 293, U20S, SW480, and Hela cells, while ER-stress was the most potent inducer of GADD34 in A549 cells. Finally, in DU451 and COS7 cells ER-stress and arsenite resulted in similar increases in GADD34 expression. In all cell lines tested, okadaic acid was the weakest GADD34-inducer. All inductions of GADD34 were blocked by treatment with actimomycin-B, indicating that transcription is upregulated by stress. These data indicate that GADD34 transcription is regulated my multiple upstream signals. We are now investigating the role of c-Myc in p38-dependent and independent GADD34 up-regulation.

In addition to studying the transcriptional up-regulation of GADD34, we have examined the degradation of GADD34. GADD34 contains 4 PEST repeats, and was predicted to be a rapidly turned over protein. Studying both endogenous and over-expressed GADD34, we determined that GADD34 was indeed rapidly turned-over, with a half-life of less than 15 minutes. Deletion analysis indicated that surprisingly, this degradation was independent of the PEST repeats. We mapped the degradation motif to amino acids 550-600, overlapping the PP1 binding motif. This motif contains several lysines that are possible ubiquitination sites. We are currently attempting to confirm that GADD34 is ubiquinated, and we are mutating the lysines to argenine. These mutations should not interfere with PP1 binding, but may produce a more stable protein. These experiments will lead to a better understanding of the dynamic expression and degradation of GADD34, and confirm that it is a tightly temporally regulated stress-response gene.

Task 1B is to evaluate the function of tagged human GADD34 overexpression. Mutations were made in GADD34 and the structure-function relationship for GADD34 mediated dephosphorylation of eIF2 alpha was established. This work has been published (Brush et al 2003). We identified a novel C-terminal domain of GADD34 called the ArgAla domain that is critical for proper interaction with PP1 and eIF2 alpha dephosphorylation. This work will then be used as the basis for studies of the mechanism of GADD34-induced apoptosis.

Task 1C is to use the substrate of the GADD34/PP1 complex as a tool to understand c-Myc-induced apoptosis. We have conducted time-course experiments monitoring the stress-induced accumulation of GADD34 and the level of eIF2 alpha phosphorylation. Using multiple cancer cell lines, we determined that GADD34 protein accumulated approximately two hours after the initiation of stress. The level of eIF2 alpha phosphorylation peaks at one hour and decreases as GADD34 accumulates. This is consistent with prior work conducted in mice (Novoa et al, 2001 and Novoa et al, 2004). These data indicate that GADD34 serves as a feedback inhibitor of translational inhibition by the stress response pathway in cancer cell lines.

The goal of **Task 2** is to understand the mechanisms of GADD34 regulation in breast cancer cells. Toward **Task 2A** we expressed GADD34 in tissue culture cells and

metabolically labeled with 32p-orthophosphatase. We have discovered novel phosphorylation sites and mapped them to the central pest-repeat region. We are currently conducting additional mutagenesis and micro-sequencing experiments to identify the specific phosphorylated residues. Once identified, we will determine the functional significance of these sites on eIF2 alpha phosphorylation and GADD34 stability.

The goal of **Task 2B** was to examine the mechanism of GADD34 subcellular localization. These experiments have been conducted and published (Brush et al, 2003). Endogenous GADD34 was shown to be localized to the endoplasmic reticulum (ER). A short hydrophobic loop in the N-terminus was shown to be necessary for GADD34 localization to the ER. GADD34 also contains putative nuclear import and export domains. Mutant forms of GADD34 that accumulate in the nucleus fail to induce eIF2 alpha dephosphorylation, indicating that proper subcellular localization of GADD34 is critical for proper function.

The goal of **Task 2C** was to study the role of Inhibitor-1 in controlling eIF2 alpha dephosphorylation. To this end we initially characterized I-1 function in both cancer cell lines and yeast. We demonstrated that I-1 inhibits eIF2 alpha dephosphorylation *in vitro* and *in vivo*. We also identified a novel domain of I-1 that is critical for *in vivo* function. This domain was characterized further as an effector domain necessary for high affinity interaction with PP1. In two recently identified but previously uncharacterized alternate splice forms of I-1, termed I-1 alpha and I-1 beta, this domain is deleted (Liu et al, 2002). We demonstrated that these splice variants are differentially expressed in mouse tissues, and over development. We also demonstrated that these splice variants are reduced in there ability to induce accumulation of phosphorylated eIF2 alpha. These studied have been submitted to JBC (Weiser et al. 2004).

Consistent with my predoctoral training we have also conducted several collaborative experiments involving the regulation of PP1, but not directly involving eIF2 alpha dephosphorylation. We have examined the role of I-1 in controlling cardiac adrenergic signaling; this work has been submitted to the Journal of Clinical Investigation (Pathak et al. 2004). Another work involving the regulation of PP1 by targeting and inhibitory subunits is in preparation (Gibbons et al. 2004). These experiments have lead to a better understanding of the mechanisms of PP1 regulation and these lessons are being applied to the study of GADD34 signaling in cancer cells. We also studied the control of Mitosis by PP1-mediated dephosphorylation of CDC25 (Margolis SS, et al, 2003). This provided a mechanistic understanding of the regulation of cell cycle checkpoints, and is of direct relevance to the study of defects in cell cycle regulation found in many human cancers. Finally, we wrote a chapter for Current Protocols in Protein Science, involving the use of protein phosphates inhibitors for the study of signaling pathways. The writing of this review and these collaborative projects have greatly aided my predoctoral training by exposing me to diverse lines of research and will aid in my future as an independent scientist.

Key Research Accomplishments:

- Demonstration of GADD34 protein accumulation following diverse stresses in human cancer cell lines
- Demonstration that GADD34 induction by arsenite is dependent of p38 MAP Kinase, but ER-stress induction of GADD34 is independent of p38 MAP Kinase
- Demonstrating that induction of endogenous GADD34 corresponds with dephosphorylation of eIF2 alpha
- Demonstrating role of Inhibitor-1 (I-1) in regulation of eIF2 alpha dephosphorylation *in vivo*
- Identification of a novel domain of I-1 required for *in vivo* function
- Demonstration of modified activity of two splice variants of I-1
- Collaborative investigation of the regulation of PP1 by targeting subunits, inhibitors, and substrate specificity

Reportable Outcomes:

Manuscripts:

Gibbons JB, **Weiser DC**, Shenolikar S. Structural Requirements for Regulation of Protein Phosphatase 1 by Endogenous Proteins and Environmental Toxins. In Preparation, 2004

Margolis SS, Walsh S, **Weiser DC**, Yoshida M, Shenolikar S, Kornbluth S. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. **EMBO J.** 2003 Nov 3;22(21):5734-45.

Pathak A, Del Monte F, Zhao W, Mitton B, Carr A, Shultz J, Hahn H, Syed F, Bodi I, **Weiser DC**, Mavila N, Jha L, O'Neal E, Qian J, Fan G, Marreez Y, McGraw D, Heist E, Guerrero J, DePaoli-Roach A, Hajjar R and Kranias EG. Enhancement of Cardiac Function and Suppression of Heart Failure Progression by Inhibition of Protein Phosphatase 1. Submitted **Journal of Clinical Investigation**, 2004

Weiser DC and Shenolikar S. Use of Protein Phosphatase Inhibitors. (2003) **Current Protocols in Protein Science** 13.10

Weiser DC, Sikes SJ, Li S, Shenolikar S. A Novel Functional Domain of Protein Phosphatase Inhibitor-1: I-1 Regulation of eIF2 alpha Dephosphorylation. Submitted **Journal of Biological Chemistry**, 2004

Poster Presentations:

Douglas Weiser, Shirish Shenolikar. Regulation of eIF2 alpha Dephosphorylation by Protein Phosphatase Inhibitor-1. Duke University Medical Center department of Pharmacology and Cancer Biology, departmental retreat, September 2003

Douglas Weiser, Shirish Shenolikar. Regulation of eIF2 alpha Dephosphorylation by Protein Phosphatase Inhibitor-1. Duke University Medical Center Graduate Student Symposium, November 2003

Gibbons JB, **Weiser DC**, Shenolikar S. Structural Requirements for Regulation of Protein Phosphatase 1 by Endogenous Proteins and Environmental Toxins. FASEB conference on Protein Phosphatases, July 2004

Conclusions:

Toward the goal of understanding GADD34-induced apoptosis we have conducted several lines of research into the function of GADD34. Prior to this reporting period we identified a novel domains of GADD34 required for PP1 binding, and another required for ER-localization. We demonstrated that correct subcellular localization was required for GADD34 function, and determined that endogenous GADD34 is primarily localized to the ER. These observations were published prior to this reporting period (Brush et al, 2003).

Since July 2003 we have examined the role of Inhibitor-1 in controlling eIF2 alpha dephosphorylation. I-1 can bind GADD34 and inhibit PP1 (Conner et al, 2001). We established that I-1 can inhibit eIF2 alpha dephosphorylation in cancer cells and yeast. We also identified a novel functional domain of I-1 required for *in vivo* function, and discovered that this domain is deleted in splice variants of I-1. These observations have been submitted to the Journal of Biological Chemistry (Weiser et al, 2004)

We have also examined the dynamic expression pattern of GADD34. We have demonstrated that GADD34 protein levels accumulate in a stress-dependent manner, and GADD34 up-regulation by arsenite treatment but not ER-stress is dependent on p38 MAP kinase activity. We have also demonstrated that GADD34 accumulation results in eIF2 alpha dephosphorylation, indicating that GADD34 is a feed-back inhibitor of stress-signaling controlled by diverse transcriptional mechanisms. We also demonstrated that GADD34 is a rapidly turned-over protein and that this degradation is independent on the PEST repeats and may involve ubiquitination of the PP1 binding domain. These experiments indicate that GADD34 accumulation is a tightly regulated phenomenon at both the transcriptional and post-translational level. The study of this mechanism may lead to a better understanding of breast cancer biology.

As part of my predoctoral training I also conducted collaborative investigations of PP1 function (Margolis et al 2003, Pathak et al, 2004 Gibbons et al, 2004) and wrote a methods review (Weiser and Shenolikar, 2003). These efforts provided an excellent opportunity to share my knowledge of PP1 regulation, acquired during my predoctoral career. They also allowed me to engage in diverse research projects.

We would consider **Task 1B, Task 2B** (Brush et al., 2003), and **Task 2C** (Weiser et al, 2004) completed and published. Our future efforts will focus on **Task 1A, Task 1C** and **Task 2A**. To this end we will determine the upstream pathways required for the induction of GADD34 protein in cancer cells. We will also focus on the protein stability of GADD34, and determine the mechanism of its degradation. Finally, we will map the novel phosphorylation sites on GADD34 and determine their functional significance.

References:

Amundson SA, Zhan Q, Penn LZ, Fornace AJ Jr. Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents. **Oncogene**. 1998 Oct 29;17(17):2149-54.

Brush MH, Weiser DC, Shenolikar S. Growth Arrest and DNA Damage-Inducible Protein GADD34 Targets Protein Phosphatase 1 Alpha to the Endoplasmic Reticulum and Promotes Dephosphorylation of the Alpha Subunit of Eukaryotic Translation Initiation Factor 2. **Mol Cell Biol**. 2003 Feb;23(4):1292-303.

Connor JH, Weiser DC, Li S, Hallenbeck JM, Shenolikar S. Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. **Mol Cell Biol**. 2001 Oct;21(20):6841-50.

Gibbons JB, Weiser DC, Shenolikar S. Structural Requirements for Regulation of Protein Phosphatase 1 by Endogenous Proteins and Environmental Toxins. In preparation 2004.

Hollander MC, Sheikh MS, Yu K, Zhan Q, Iglesias M, Woodworth C, Fornace AJ Jr. Activation of Gadd34 by diverse apoptotic signals and suppression of its growth inhibitory effects by apoptotic inhibitors. **Int J Cancer**. 2001 Feb 20;96(1):22-31.

Liu HT, Lin TH, Kuo HC, Chen YC, Tsay HJ, Jeng HH, Tsai PC, Shie FK, Chen JH, Huang HB. Identification of the alternative splice products encoded by the human protein phosphatase inhibitor-1 gene. **Biochem Biophys Res Commun**. 2002 Mar 15;291(5):1293-6.

Margolis SS, Walsh S, Weiser DC, Yoshida M, Shenolikar S, Kornbluth S. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. **EMBO J**. 2003 Nov 3;22(21):5734-45.

Novoa I, Zhang Y, Zeng H, Jungreis R, Harding HP, Ron D. Stress-induced gene expression requires programmed recovery from translational repression. **EMBO J**. 2003 Mar 3;22(5):1180-7.

Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. **J Cell Biol**. 2001 May 28;153(5):1011-22

Pathak A, Del Monte F, Zhao W, Mitton B, Carr A, Shultz J, Hahn H, Syed F, Bodi I, Weiser DC, Mavila N, Jha L, O'Neal E, Qian J, Fan G, Marreez Y, McGraw D, Heist E, Guerrero J, DePaoli-Roach A, Hajjar R and Kranias EG. Enhancement of Cardiac Function and Suppression of Heart Failure Progression By Inhibition of Protein Phosphatase 1. Submitted **Journal of Clinical Investigation**, 2004

Sinha R, Kiley SC, Lu JX, Thompson HJ, Moraes R, Jaken S, Medina D. Effects of methylselenocysteine on PKC activity, cdk2 phosphorylation and gadd gene expression in synchronized mouse mammary epithelial tumor cells. **Cancer Lett.** 1999 Nov 15;146(2):135-45.

Weiser DC and Shenolikar S. Use of Protein Phosphatase Inhibitors. (2003) **Current Protocols in Protein Science** 13.10

Weiser DC, Sikes SJ Li S, Shenolikar S. A Novel Functional Domain of Protein Phosphatase Inhibitor-1: I-1 Regulation of eIF2 alpha Dephosphorylation. Submitted **Journal of Biological Chemistry**, 2004